SHORT COMMUNICATION

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## Employment of molecular markers to develop tetraploid "supermale" asparagus from andromonoecious plants of the landrace 'Morado de Huétor'

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## Abstract

The aim of this work was the development of new "supermale" genotypes retaining the highest genetic diversity possible of the tetraploid asparagus landrace 'Morado de Huétor'. The elite andromonoecious male HT664 of the 'Morado de Huétor' asparagus landrace and the andromonoecious hybrid male HC027, obtained by crossing between this landrace and a commercial cultivar of *Asparagus officinalis*, were selected for self-pollination to produce possible "supermales" with genes of 'Morado de Huétor' (SMHT). To confirm the hybrid nature of HC027, we characterized this genotype with EST-SSR (Expressed Sequence Tag-derived Simple Sequence Repeats) markers. We also adopted the sex-linked marker Asp1-T7 for sex determination in 'Morado de Huétor' and the resulting hybrids between this landrace and other commercial cultivars. Asp1-T7 marker was used for the selection and genotyping of SMHT. "All-male" cultivars with a different genetic background can be generated by crossing females with these new "supermale" genotypes, and the agronomical traits of these new cultivars would be very different from the "all-male" cultivars currently available in the market, making them extremely interesting for asparagus breeding programs.

Additional key words: all-male cultivars; Asparagus landrace; EST-SSRs; hybrids; S-males; sex-linked marker.

Asparagus officinalis L., is a diploid dioecious species with a chromosome number of 2n = 2x = 20. It is an important crop plant that is grown in temperate climate regions worldwide. Most of the commercial varieties are diploid and derive from the old cultivar 'Purple Dutch' (Ellison, 1986; Geoffriau *et al.*, 1992). Because of their common origin, the current commercial cultivars are thought to have a narrow genetic basis (Brettin & Sink, 1992; Geoffriau *et al.*, 1992; Lallemand *et al.*, 1994; Khandka *et al.*, 1996; Moreno *et al.*, 2006). The use of asparagus landraces in breeding programs can enlarge the gene pool of cultivated asparagus. 'Morado de Huétor' is a Spanish tetraploid landrace (2n = 4x = 40) cultivated in the south of Spain, and seems to be the result of a hybridization between A. officinalis and Asparagus maritimus Mill (Moreno et al., 2008a). This landrace presents higher and different variability than commercial varieties (Geoffriau et al., 1992; Moreno et al., 2006). Hence, the different genetic background makes this landrace a valuable genetic resource for asparagus breeding programs because it can be employed to widen the asparagus genetic base and to introgress traits. In A. officinalis populations, it is possible to find plants with different ploidy levels, in spite of their diploid genome, which is a result of the formation of unreduced gametes (2n) (Camadro, 1992, 1994). The cross between these tetraploid and diploid plants could generate triploid hybrids (Hasegawa et al., 1987; Skiebe et al., 1991; Ozaki et al., 2004). Hybrids between diploid plants

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Abbreviations used: EST-SSR (expressed sequence tag-simple sequence repeats); FCM (flow cytometry); SMHT ("supermales" from 'Morado de Huétor').

from commercial cultivars and tetraploid 'Morado de Huétor' have been developed and can be used to widen the genetic pool of commercial asparagus cultivars (Moreno *et al.*, 2010). Recently, triploid hybrids have been successfully employed in the development of diploid plants with genetic variability introgressed from 'Morado de Huétor' (Castro *et al.*, 2014).

Sex in asparagus is determined by a dominant gene, M (Flory, 1932), located on the homomorphic chromosome pair L5 (Löptien, 1979). In diploid asparagus, the female genotypes are homozygous recessive (mm) and the male genotypes are heterozygous (Mm), resulting in the ratio 1:1 (male: female) in traditional cultivars. This ratio is conserved in the tetraploid landrace 'Morado de Huétor', suggesting that the male genotypes of this population are Mmmm and female genotypes are mmmm (Moreno et al., 2008b). Male plants sometimes bear bisexual flowers, and these male genotypes are named andromonoecious. Self-pollination of those flowers can produce "supermales" (MM). Crosses between "supermales" or "s-males" and female genotypes will raise a progeny consisting exclusively of male plants (Sneep, 1953). Male plants show advantages over female plants. The male plants will never produce seeds, avoiding the growth of the seeds into weeds. In addition, their yield, longevity and tolerance to diseases are higher than in females (Ellison, 1986; López-Anido & Cointry, 2008). Hence, the "supermales" are used to develop "allmale" cultivars (Elisson & Kinelski, 1985; Ellison et al., 1990). At present, the development of "all-male" hybrid cultivars is a main objective in many asparagus breeding programs, and some diploid "all-male" cultivars have been released. Using an anther culture can be an alternative technique to obtain "supermales". It has been successfully employed in both diploid and tetraploid cultivars, obtaining haploid and diploid plants, respectively (Dorè, 1974, 1990; Qiao & Falavigna, 1990; Riccardi et al., 2011). The haploid (M) and diploid (Mm) males obtained in the anther culture can produce, through polyploidization, di-haploid (MM) and didiploids (MMmm). The first can be considered a "supermale" and the second a near-supermale because would give a progeny with a ratio of 5 males: 1 female. Therefore, the process to obtain a tetraploid "supermale" would be longer than a diploid one.

Asparagus is a dioecious species; thus, the differentiation between male or female plants requires waiting until flowering, which takes approximately 1-2 years after transplantation (Sneep, 1953). Furthermore, "supermales" (*MM*) cannot be differentiated phenotypically from the males (Mm), even at flowering. Therefore, to detect "supermale" genotypes using classic methods, it is necessary to follow a long program of test-crosses, which will consume at least two working years to characterize the progenies. The use of a sex-linked marker can differentiate the sex of the plants at an early stage, shortening the time necessary to characterize the progenies. Jamsari et al. (2004) developed a dominant sex-linked marker (Asp1-T7) for a single commercial cultivar. Nakayama et al. (2006) adapted this marker for all commercial cultivars, and Kubota et al. (2012) showed that this sex-linked marker can be used to distinguish between male (present band) and female (absent band) genotypes in species closely related to A. officinalis. A co-dominant, sex-linked marker would allow direct identification of "supermales" without test crosses; however, to date, there are no validated markers with these characteristics.

One of the objectives in our breeding program is developing tetraploid "all-male" cultivars using the asparagus tetraploid landrace 'Morado de Huétor' to introduce new genetic variability in the cultivated varieties. In this work, we have opted to develop "supermales" through the self-pollination of andromonoecious plants of 'Morado de Huétor' instead of the anther culture to maintain the highest genetic variability possible in these "supermales". We also report the application of the sex-linked marker Asp1-T7 in identifying male 'Morado de Huétor' plants, and decreasing the time necessary to detect a "supermale" using it. This procedure sets the conditions for "supermale" development in this landrace.

Two tetraploid andromonoecious plants (HT664 and HC027) were selected to be self-pollinated to generate "supermales" from 'Morado de Huétor' (SMHT). These andromonoecious plants derived from initial crosses made to improve the agronomical traits of the andromonoecious plants that were used for the selfpollination. HT664 was derived from a cross between an elite tetraploid female from our collection (HT069) and a tetraploid andromonoecious plant found in a 'Morado de Huétor' population (HT225). HT664 was selected because it was the andromonoecious plant with the best agronomical traits among the progeny obtained from the cross. HC027 was derived from a cross between an elite tetraploid female (HT252) and a diploid andromonoecious plant (CM029) found in a field plot of commercial cultivars. HC027 was selected because it was the only tetraploid andromonoecious plant generated in the cross.

The andromonoecious genotypes HT664 and HC027 were bagged and self-pollinated by hand. The progenies obtained (SMHT) were grown in a field plot at the University of Córdoba (Spain). After two years, the progenies were phenotyped for sex determination according to their floral morphology. Six male nonandromonoecious plants from these progenies were crossed with a female plant (mmmm). Their progenies were genotyped for the sex-linked marker, Asp1-T7, to identify the male parental genotype. Two years later, they were phenotyped for plant sex according to their floral morphology. Chi-square tests, with one degree of freedom and  $\alpha = 0.05$ , were performed to analyze the male:female progeny ratio for goodness of fit to expected ratios. SMHT6, SMHT7 and SMHT8 were obtained by the self-pollination of HT664, while SMHT10, SMHT13 and SMHT 14 were obtained by the self-pollination of HC027.

Total genomic DNA of each plant was extracted from 1-g of shoot tips from young spears following a modified CTAB extraction protocol described by Torres *et al.* (1993).

To determine the applicability of the sex-linked marker Asp1-7 in the landrace 'Morado de Huétor', we used four males and four females selected at random from the asparagus germplasm collection established by Moreno et al. (2008b), as well as four males and four females from different diploid commercial cultivars as positive controls. PCR was conducted with the primers designed by Nakayama et al. (2006) (Asp1-T7spf and Asp1-T7spr). A partial sequence (AODEF-Taq1) of 97 bp within AODEF, a Bfunctional MADS-box gene (accession no. AB180962; Park et al., 2003) was used as a positive control for DNA quality to discard the presence of false negatives in the amplification of the sex-linked marker, Asp1-T7. This sequence was amplified using the primers designated by Horiuchi et al. (2011) (AODEF-Taq1F and AODEF-Taq1R). We followed the protocols of Nakayama et al. (2006) and Horiuchi et al. (2011) for PCR procedures.

HC027 and its parental lines (HT252 and CM029) were analyzed using EST-SSRs markers to check its hybrid nature. Four EST-SSR markers (TC1, TC9, AG3, AG10) previously developed by Caruso *et al.* (2008) were employed in this study. Forward primers were synthesized with either the fluorescent dye 6FAM (TC1, AG3) or HEX (TC9, AG10) (Applied Biosystems) at the 5' ends. Amplification of these markers was performed as in Caruso *et al.* (2008). The PCR

products were separated using an automated capillary sequencer (ABI 3130 Genetic Analyzer; Applied Biosystems/HITACHI, Madrid, Spain) in the Unit of Genomics of the Central Research Support Service at the University of Córdoba.

In this study HC027 is the only tetraploid and romonoecious plant generated by the cross between the andromonoecious diploid plant CM029 and the tetraploid female HT252. The remaining plants obtained from this cross were triploid, as expected from a cross between a diploid and a tetraploid plant. The ploidy level of HC027 and the other plants obtained in this cross was determined by flow cytometry (FCM). The amplifications of the four EST-SSRs in HC027 showed the presence of alleles specific to each parental genotype. These results indicate that the tetraploid andromonoecious HC027 is a hybrid, with half of the genetic background coming from 'Morado de Huétor' (HT252) and the other half coming from a commercial cultivar (CM029). Therefore, the tetraploid HC027 could be generated as a result of the formation of an unreduced gamete in CM029. The formation of unreduced gametes in asparagus has been reported by Camadro (1992, 1994). Thus, we could discard a possible contamination with other pollen and confirm the hybrid nature of the tetraploid andromonoecious HC027.

All male samples showed identical amplicons of 308 bp for Asp1-T7 marker, without differences between 'Morado de Huétor' and commercial cultivars, while in the female samples, the amplicon was not observed (Fig. 1). The primers designed by Horiuchi et al. (2011) amplified a fragment of 97 bp in all our samples, indicating that there were no false negatives in the amplification produced by Asp1-T7. These results indicate that the sex-linked marker Asp1-T7 can be successfully used to determine sex in the tetraploid landrace 'Morado de Huétor', shortening the time necessary to detect "supermales". Our results show that Asp1-T7 could be a good tool to analyze the male/female segregation in an early developmental stage of the progenies from the test-cross, saving time by avoiding the delay of 1 to 2 years until plant flowering.

Selfing of HT644 and HC027 produced progenies that fit the expected segregation ratio of 3 male:1 female for the andromonoecious genotype *Mmmm* (Table 1). In the tetraploid landrace 'Morado de Huétor', the sex ratio 1(male):1(female) was conserved, suggesting that the males are *Mmmm* and the females are *mmmm* (Moreno *et al.*, 2008b). If an *MMmm* 



**Figure 1.** Amplification of the sex-linked marker Asp1-T7 with DNA isolated from four males and four females randomly selected from the germplasm collection of 'Morado de Huétor' (Moreno *et al.*, 2008b). Four male and four female commercial cultivars were included as controls. DNA marker is Hyperladder<sup>TM</sup> V.

**Table 1.** Segregation of plant sex in the progenies obtainedafter self-pollination of HT664 and HC027 based on thephenotypic characterization of flowers

Plant	Male	Female	Total	$\chi^{2}_{3:1}$	Genotype	
HT644	23	6	29	0.29	$Mmmm^1$	
HC027	31	8	39	0.42	$Mmmm^1$	

<sup>1</sup> There were no statistically significant differences between the ratio of male: female observed and the ratio expected to a *Mmmm* genotype when tested with  $\chi^2$ ,  $\alpha = 5\%$ .

genotype appears in a tetraploid population and it is crossed with a female *mmmm*, a segregation ratio of 1 *MMmm*:4 *Mmmm* will be expected among the male progeny obtained from the cross. If an *MMmm* genotype appears in a tetraploid population, a ratio of 1 *MMmm*:4 *Mmmm* will be expected among the male progeny obtained after crossing with females (mmmm). Therefore, after several generations, the MMmm genotype will practically disappear. Therefore, the male plants obtained in the self-pollination of HT664 and HC027 (SMHT plants) would be Mmmm or MMmm. To detect the MMmm genotypes, three male plants from every self-pollination (SMHT6, SMHT7 and SMHT8 from HT664 and SMHT10, SMHT13 and SMHT14 from HC027) were selected to carry out a test-cross with a female plant (mmmm). The progenies generated in these test-crosses were analyzed with the sex-linked marker Asp1-T7 in order to genotype the SMHT plants. The data were tested for goodness of fit to either 1:1 (male parent Mmmm) or 5 (male):1 (female) (male parent *MMmm*) segregation ratios using chi square tests ( $\chi^2$ ) (Table 2). The progenies from SMHT8, SMHT10 and SMHT14 showed a segregation of 1 male:1 female, indicating that these three males

**Table 2**. Sex determination of SMHT ("supermales" from 'Morado de Huétor') progenies using the sex-linked marked Asp1-T7 and the phenotypic analysis of flowering plants

Plant	Analyzed with Asp1-T7			Phenotypical analysis of flowers				— Agreement	Genotype	
SMHT	Female	Male	Total	$\chi^2$	Female	Male	Total	$\chi^2$	Agreement	Genotype
6	8	23	31	1.81	3	17	20	0.06	Yes	$MMmm^1$
7	8	23	31	1.81	6	21	27	0.33	Yes	$MMmm^1$
8	10	11	21	0.05	7	13	20	1.80	Yes	$Mmmm^1$
10	14	12	26	0.15						$Mmmm^1$
13	3	27	30	0.96	3	25	28	1.00	Yes	$MMmm^1$
14	17	13	30	0.53	15	11	26	0.61	Yes	$Mmmm^1$

<sup>1</sup> There were no statistically significant differences between the male-female ratio observed and the ratio expected to a *Mmmm/MMmm* genotype when tested with  $\chi^2$ ,  $\alpha = 5\%$ . —: no data.

have the genotype *Mmmm*. The progenies from SMHT6, SMHT7 and SMHT13 fit the segregation ratio of 5 male:1 female, confirming that these three males (SMHT6, SMHT7 and SMHT13) have the geno-type *MMmm*; thus, they were named "near-supermales". To confirm the genotype of the selected SMHT plants, we phenotyped the progeny obtained from each test-cross as male or female when they flowered (two years after planting). The difference between the total numbers of plants genotyped with the marker Asp1-T7 and the total numbers of plants before flowering.

In summary, in this study genotypes MMmm have been early identified using Asp1-T7 marker, these plants may be used also to develop super-males by anther culture. However, it could be interesting to use MMmm genotypes to develop hybrid varieties with a high percentage of male plants and a different genetic background. The agronomical traits of these new cultivars would be very different from the "all-male" cultivars currently available in the market, making them extremely interesting for asparagus breeding programs. Asp1-T7 is a dominant marker associated with the male sex; therefore, this marker cannot distinguish between Mm and MM in diploid genotypes or Mmmm, *MMmm*, *MMMm* and *MMMM* in tetraploid genotypes. More effort should be done to develop a co-dominant marker which would allow us to detect MM or MMMM genotypes without requiring test-crossing with females.

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## References

- Brettin TS, Sink C, 1992. Allozyme variation and genetics in asparagus. J Hered 83: 383-386.
- Camadro EL, 1992. Cytological mechanism of 2n microspore formation in garden asparagus. HortScience 27(7): 831-832.
- Camadro EL, 1994. Second meiotic restitution (SDR) 2n pollen formation in diploid and hexaploid species of *Asparagus*. Genet Resour Crop Evol 41: 1-7.
- Caruso M, Federici CT, Roose ML, 2008. EST-SSR markers for asparagus genetic diversity evaluation and cultivar identification. Mol Breed 21: 195-204.

- Castro P, Rubio J, Gil J, Moreno R, 2014. Introgression of new germplasm in current diploid cultivars of garden asparagus from a tetraploid Spanish lancrace "Morado de Huétor". Sci Hortic 168: 157-160.
- Doré C, 1974. Production de plantes homozygotes mâles et femalles à partir d'anthères d'asperge cultivées in vitro. C R Acad Sci 278: 2135-2138.
- Doré C, 1990. Asparagus anther culture and field trials of dihaploids and F1 hybrids. In: Bajaj YPS (Ed) haploids in crop improvement. I. Biotechnology in agriculture and forestry, vol. 12. Springer-Verlag, Berlin (Germany), pp: 322-345.
- Ellison JH, 1986. Asparagus breeding. In: Breeding vegetable crops (Bassett MJ, ed). AVI Publishing Co., Westport (Ireland), pp: 521-569.
- Ellison JH, Kinelski JJ, 1985. Jersey Giant, an all-male asparagus hybrid. HortScience 20: 1141.
- Ellison JH, Garrison SA, Kinelski JJ, 1990. Male asparagus hybrids-Jersey Gem, Jersey General, Jersey King, Jersey Knight, and Jersey Titan. HortScience 25: 816-817.
- Flory WS, 1932. Genetic and cytological investigations on *Asparagus officinalis* L. Genet 17: 432-467.
- Geoffriau E, Denoue D, Rameau C, 1992. Assessment of genetic variation among asparagus (Asparagus officinalis L.) population and cultivars: agromorphological and isozymatic data. Euphytica 61: 169-179.
- Hasegawa S, Taniguchi Y, Okimori A, Kakehi M, 1987. Studies on polyploidy breeding in asparagus (*Asparagus officinalis* L.) 2. On the characteristics of triploid. Bull Hiroshima Prefect Agric Exp Stn 50: 75-80.
- Horiuchi K, Adachi Y, Kasai N, Yamagishi M, Masuda K, 2011. Identification of homozygous male plants by quantitative analysis of a nucleotide sequence linked to the sex-determination locus in *Asparagus officinalis* L. J Japan Soc Hort Sci 80 (3): 308-313.
- Jamsari A, Nitz I, Reamon-Büttner SM, Jung C, 2004. BACderived diagnostic markers for sez determination in asparagus. Theor Appl Genet 108: 1140-1146.
- Khandka DK, Nejidat A, Golan-Goldhirsh A, 1996. Polymorphism and DNA markers for asparagus cultivars identified by random amplified polymorphic DNA. Euphytica 87: 39-44.
- Kubota S, Konno I, Kanno A, 2012. Molecular phylogeny of the genus *Asparagus* (Asparagacea) explains interspecific crossability between the garden asparagus (*A. officinalis*) and other *Asparagus* species. Theor Appl Genet 124: 345-354.
- Lallemand J, Briand F, Breuils F, Denoue D, Rameau C, 1994. Identification of asparagus varieties by isozyme patterns. Euphytica 79: 1-4.
- López-Anido F, Cointry E, 2008. Asparagus. In: Vegetables II: Fabaceae, Liliaceae, Umbelliferae, and Solanaceae (Prohens J, Nuez F, eds). Series: Handbook of plant breeding. Springer, NY, (USA), pp: 87-119.
- Löptien H, 1979. Identification of the sex chroomosome pair in asparagus (*Asparagus officinalis* L.). Z Pflanzenzücht 82: 162-173.
- Moreno R, Espejo JA, Cabrera A, Millan T, Gil J, 2006. Ploidic and molecular analysis of 'Morado de Huetor'

asparagus (*Asparagus officinalis* L.) population; a Spanish tetraploid landrace. Genet Resour Crop Evol 53: 729-736.

- Moreno R, Espejo JA, Cabrera A, Gil J, 2008a. Origin of tetraploid cultivated asparagus landraces inferred from nrDNA ITS polymorphisms. Ann Appl Biol 153: 233-241.
- Moreno R, Espejo JA, Moreno MT, Gil J, 2008b. Collection and conservation of "Morado de Huetor" Spanish tetraploid asparagus landrace. Genet Resour Crop Evol 55: 773-777.
- Moreno R, Espejo JA, Gil J, 2010. Development of triploid hybrids in asparagus breeding employing a tetraploid landrace. Euphytica 173: 369-375.
- Nakayama H, Ito T, Hayashi Y, Sonoda T, Fukuda T, Ochiai T, Kameya T, Kanno A, 2006. Development of sex-linked primers in garden asparagus (*Asparagus officinalis* L.). Breed Sci 56: 327-330.
- Ozaki Y, Narikiyo K, Fujita C, Okubo H, 2004. Ploidy variation of progenies from intra- and inter-ploidy crosses with regard to trisomic production in asparagus (*Aspara*gus officinalis L.). Sex Plant Reprod 17: 157-164.

- Park JH, Ishikawa Y, Yoshida R, Kanno A, Kameya T, 2003. Expression of AODEF, a B-functional MADS-box gene, in stamens and inner tepals of the dioecious species *Asparagus officinalis* L. Plan Mol Biol 51: 867-875.
- Qiao Y, Falavigna A, 1990. An improved *in vitro* anther culture method for obtaining doubled-haploid clones of *Asparagus*. Acta Hort 271: 145-150.
- Riccardi P, Casali PE, Mercati F, Falavigna A, Sunseri F, 2011. Genetic characterization of asparagus doubled haploids collection and wild relatives. Sci Hort 130: 691-700.
- Skiebe K, Stein M, Gottwald J, Wolterstorff B, 1991. Breeding of polyploid asparagus (*Asparagus officinalis* L.). Plant Breed 106: 99-106.
- Sneep J, 1953. The significance of andromonoecy for the breeding of *Asparagus officinalis* L. Euphytica 2: 89-95.
- Torres AM, Weeden NF, Martín A, 1993. Linkage among isozyme, RFLP and RAPD markers in *Vicia faba*. Theor Appl Genet 85: 937-945.